Monoclonal Antibodies against Chicken Type IV and V Collagens: Electron Microscopic Mapping of the Epitopes after Rotary Shadowing

RICHARD MAYNE, HANNA WIEDEMANN, MICHAEL H. IRWIN, RALPH D. SANDERSON, JOHN M. FITCH, THOMAS F. LINSENMAYER, and KLAUS KÜHN

Departments of Anatomy and Biochemistry, University of Alabama in Birmingham, Birmingham, Alabama 35294; Max Plack Institute of Biochemistry, D-8033 Martinsried, Munich, Federal Republic of Germany; Department of Anatomy and Cellular Biology, Tufts University, Boston, Massachusetts 02111; and Developmental Biology Laboratory, Massachusetts General Hospital, Boston, Massacchusetts 02114

ABSTRACT The location of the epitopes for monoclonal antibodies against chicken type IV and type V collagens were directly determined in the electron microscope after rotary shadowing of antibody/collagen mixtures. Three monoclonal antibodies against type IV collagen were examined, each one of which was previously demonstrated to be specific for only one of the three pepsin-resistant fragments of the molecule. The three native fragments were designated $(F1)_2F2$, F3, and 7S, and the antibodies that specifically recognize each fragment were called, respectively, IA8, IIB12, and ID2. By electron microscopy, monoclonal antibody IA8 recognized an epitope located in the center of fragment $(F1)_2F2$ and in tetramers of type IV collagen at a distance of 288 nm from the 7S domain, the region of overlap of four type IV molecules. Monoclonal antibody IIB12, in contrast, recognized an epitope located only 73 nm from the 7S domain. This result therefore provides direct visual evidence that the F3 fragment is located closest to the 7S domain and the order of the fragments must be 7S-F3-$(F1)_2F2$. The epitope for antibody ID2 was located in the overlap region of the 7S domain, and often several antibody molecules were observed binding to a single 7S domain. The high frequency with which antibody molecules were observed to bind to fragments of type IV collagen suggests that there is a single population of type IV molecules of chain organization $[\alpha1(IV)]_2\alpha2(IV)$, and that four identical molecules must form a tetramer that is joined in an antiparallel manner at the 7S domain.

The monoclonal antibodies against type V collagen, called AB12 and DH2, were both found to recognize epitopes close to one another, the epitopes being located 45–48 nm from one end of the type V collagen molecule. The significance of this result still remains uncertain, but suggests that this site is probably highly immunoreactive. It may also be related to the specific cleavage site of type V collagen by selected metalloproteinases and by α-thrombin. This cleavage site is also known to be located close to one end of the type V molecule.

Monoclonal antibodies of high affinity prepared against the different types of collagen potentially have several advantages when compared to conventionally prepared polyclonal antibodies (reviewed in reference 1). Several groups have now described the preparation of monoclonal antibodies of high specificity for each of the five major collagen types (2–9). In previous experiments, we have prepared monoclonal antibodies that specifically recognized epitopes present in native molecules of chicken type IV collagen (10, 11) and type V collagen (12). These antibodies are now being used as reagents to investigate the macromolecular organization and function of these collagens in several tissues including lens capsule (13),

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cornea (14), and skeletal muscle (15). For type IV collagen, three monoclonal antibodies were selected, each one recognizing an epitope present in only one of the three pepsin-resistant fragments of the molecule (10, 11). Previously, these three fragments were designated $(F1)_2F2$, F3, and 7S (16, 17). In the present series of experiments, the direct localization of the epitope for each of the three monoclonal antibodies was determined in the electron microscope after rotary shadowing. By examining type IV collagen molecules that contained both the intact triple helical domain and the cross-linking site between four type IV molecules called 7S (18, 19), it proved possible to obtain an "immunomap" of the location of the three pepsin-resistant fragments. These results greatly strengthen earlier conclusions that the order of the pepsin-resistant domains of chicken type IV collagen is 7S-F3-$(F1)_2F2$ (11, 17), and that in tissues there is a single type IV collagen molecule of chain organization $[\alpha1(IV)]_2\alpha2(IV)$ (16, 20). For type V collagen, the two monoclonal antibodies that we have previously characterized (12) were found to "immunomap" to epitopes close to one another and located 45–48 nm from one end of the molecule.

MATERIALS AND METHODS

Isolation and Purification of Collagens: Types IV and V collagens were isolated after limited pepsin digestion of chicken gizzards and fractionated by differential salt precipitation first in acidic and then in neutral conditions (16). Further purification of the fragment $(F1)_2F2$ was achieved by CM-cellulose chromatography of the type IV collagen fraction in nondenaturing conditions (16), followed by molecular sieve chromatography (Bio-Gel A-15m [Bio-Rad Laboratories, Richmond, CA]) at 4°C (10). Isolation of a high molecular weight fraction of type IV fragments enriched in 7S and F3 domains was achieved by molecular sieve chromatography (Bio-Gel A-15m) after denaturation of the sample at 45°C for 30 min (16, 17). To isolate a type IV collagen preparation in which 7S domains with triple-helical arms the full length of 360 nm could be observed after rotary shadowing, we obtained gizzards from chickens made lathyritic by providing β-aminopropionitrile (0.025%) in the drinking water for 4–5 wk. After initial washings of gizzard homogenates with 1 M NaCl, pH 7.4, followed by 0.5 M HAc, limited pepsin digestion was performed once at a concentration of 100 μg/ml in 0.5 M HAc, and the pepsin inactivated at pH 8.0, as described previously (16). To separate type IV and type V collagens from interstitial collagens, we collected the precipitate during differential salt precipitation between 0.65 M NaCl, 0.5 M HAc and 1.2 M NaCl, 0.5 M HAc. Type IV collagen was then separated from type V collagen and any remaining interstitial collagens by CM-cellulose chromatography in nondenaturing conditions (16), desalted by dialysis against 0.1 M HAc and lyophilized.

Preparation of Hybridomas and Production of Monoclonal Antibodies: The preparation and characterization of the five hybridomas used in this publication have been described in detail elsewhere: antibody IA8 against the $(F1)_2F2$ fragment of type IV collagen in reference 10, antibodies ID2 and IIB12 against the 7S and F3 fragments, respectively, of type IV collagen in reference 11, and antibodies DH2 and AB12 against type V collagen in reference 12. In all cases, female SJL/3 mice were immunized with the appropriate collagen, and fusion of splenocytes was performed with NS1 myeloma cells from which hybridomas of the desired characteristics were subsequently selected (1). All five antibodies were of the IgG class (10–12).

Purification of Monoclonal Antibodies: To prepare and purify large amounts of antibody, we injected 5–10-×-10^6 hybridoma cells into the peritoneal cavity of athymic nude mice (nu/nu). After 2–3 wk, ascites fluid was harvested, briefly centrifuged, and protease inhibitors added to the supernatant. Immunoglobulins were precipitated from the supernatant with ammonium sulfate (50% of saturation, 4°C). After standing for 1 h, the precipitate obtained by centrifugation (48,000 *g*, 30 min, 4°C) was redissolved in 0.01 M phosphate buffered saline (PBS) (pH 7.2) and a second ammonium sulfate precipitate (50% of saturation) performed. Immunoglobulins were dissolved in 0.01 M PBS, and dialyzed extensively against PBS before being stored at 4°C. Each immunoglobulin preparation was characterized by SDS PAGE using a 5–10% gradient slab gel without the inclusion of urea (21). Further purification of each antibody was performed by affinity chromatography using a Sepharose-4B column to which the appropriate collagen had been coupled. For antibody IA8, an affinity column of unfractionated type IV was used, whereas for antibodies ID2 and IIB12 an affinity column was prepared with a high molecular weight fraction of type IV collagen isolated after molecular sieve chromatography (Bio-Gel A-15m) and enriched in 7S and F3 domains (11, 17). For purification of antibodies DH2 and AB12, we prepared a type V collagen affinity column. For each affinity column Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ) was first activated with CNBr as described by Furthmayr (22). The activated gel was mixed with an equal volume of collagen solution (5 mg/ml, dissolved in 0.1 M calcium acetate, pH 8.8) and shaken for 24 h at 4°C. The gel was washed once with 0.1 M calcium acetate, pH 8.8, and then resuspended in an equal volume of 0.2 M calcium acetate containing 1% ethanolamine, pH 8.8, and shaken for 6 h at 4°C to block unreacted sites. Subsequently, the gel was washed with 0.1 M calcium acetate, pH 8.0, followed by 1 M HAc, 0.15 M NaCl until a pH of 3.0 was reached. The gel was then washed with 0.1 M Tris to a pH of greater than 7.0. Finally, the gel was washed with 0.1 M PBS, pH 7.4, containing 0.02% sodium azide and stored at 4°C. The efficiency of coupling was always >50%. Affinity columns (0.5 × 12 cm) were prepared for each gel, and ammonium sulfate precipitates from ascites fluid were redissolved in PBS and passed over the appropriate column. Material that was retained on the column was eluted with 3 M KSCN, 0.05 M PBS, pH 6.0, dialyzed immediately against PBS and concentrated by ultrafiltration. Protein assays were performed by the method of Lowry (23), and ELISAs for antibody activity were carried out with a Hybridoma Screening Kit (Bethesda Research Laboratories, Gaithersburg, MD), as described previously (10).

Fig. 1 shows the purification of monoclonal antibody DH2 against type V collagen and was representative of the purifications achieved. Two light chains were present in the ammonium sulfate precipitate of the immunoglobulins from ascites fluid (Fig. 1, lanes *1* and *2*). However, only one of these chains was present in the immunoglobulin fraction which was not retained on the type V affinity column (Fig. 1, lanes *3* and *4*). This chain is therefore derived from the NS1 myeloma that can synthesize its own light chains and will incorporate them into intact immunoglobulin molecules (24). Both light chains were present in the retentate from the column (Fig. 1, lanes *5* and *6*), but the faster migrating light chain from the splenocyte predominated. The immunoglobulin fractions purified by this procedure therefore contain some hybrid molecules in which one light chain is derived from the NS1 myeloma and one light chain is derived from the splenocyte. Such hybrid antibody molecules were observed for all five monoclonal antibodies that were purified, although the relative proportions of the two light chains varied.

Rotary Shadowing of Collagen/Antibody Binding: The procedures used for rotary shadowing with platinum and subsequent electron

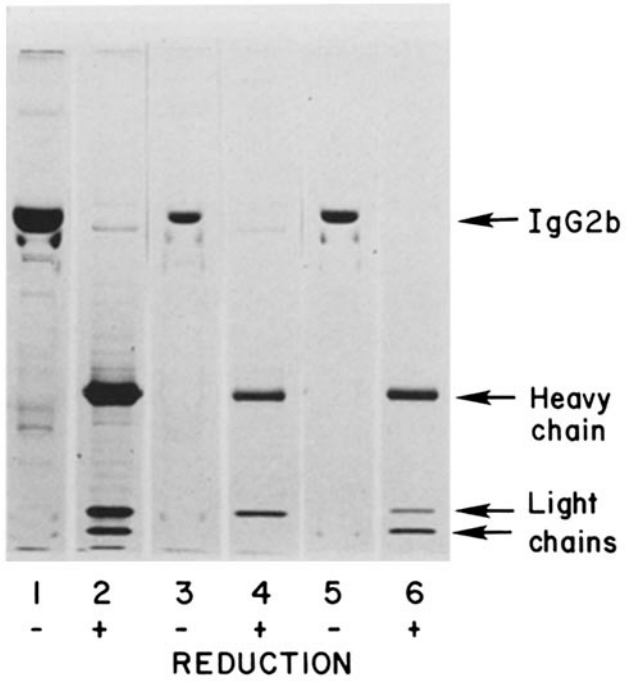

FIGURE 1 SDS PAGE (5–10% gradient gel) of monoclonal antibody DH2 before and after chromatography on a type V affinity column. (Lanes *1* and *2*) The ammonium sulfate precipitate from ascites fluid. (Lanes *3* and *4*) The eluate from the column. (Lanes *5* and *6*) The retentate that was subsequently eluted with 3 M KSCN, pH 6.0.

microscopy and photography were as described previously (19), except that a mixture of the collagen and antibody solutions were first dialyzed overnight against 0.2 M ammonium bicarbonate before spraying. Lengths of collagen molecules and the location of antibody binding sites were measured using a Science Accessories Graf Pen 3 sonic digitizer (resolution 0.1 mm) interfaced with a Hewlett Packard 9825A programable calculator. As a control for nonspecific binding of antibody molecules during rotary shadowing, both type IV spiders and type V were shadowed in the presence of an IgG1 monoclonal antibody 317 against human plasma fibronectin (25). No specific localization of the antifibronectin antibody to either type IV or type V collagen molecules was observed.

RESULTS

Localization of the Epitopes for Monoclonal Antibodies to Type IV Collagens

ANTIBODY IA8: Fig. 2 shows an electron micrograph after rotary shadowing of antibody IA8 interacting with a highly purified preparation of the fragment $(F1)_2F2$. The antibody was observed to bind to the center of all $(F1)_2F2$ fragments. The length of $(F1)_2F2$ was measured to be 149 ± 7 nm, this value comparing closely to our previous length of 147 nm for this fragment (17). For mixtures of $(F1)_2F2$ fragments and antibody IA8, we found that 91% (149 fragments examined) possessed an antibody molecule located in the center; therefore, we propose that is the location of the epitope.

We then used the antibody IA8 to locate its epitope within larger fragments of type IV collagen in which the 7S domain and the full length of the triple helix were present. Fig. 3 shows rotary shadowing performed with a mixture of antibody IA8 and a type IV collagen preparation isolated from lathyritic chicken gizzards with pepsin digestion performed at a concentration of only 100 μg/ml. We observed numerous 7S domains (Fig. 3, arrows) from which one or more arms of collagen triple helix extended. In each of the longer arms the binding of an antibody molecule was observed to occur near the end. No evidence was found for specific binding of antibody molecules elsewhere along the collagen triple helix or at the 7S domain. For one of the 7S domains shown in Fig. 3 three full length arms of type IV collagen were observed, each one of which possessed a IA8 antibody bound to the same site. Free $(F1)_2F2$ fragments were also often observed with an antibody molecule binding to the center of the fragment (Fig. 3). Measurement of the length of the longer arms from the overlap of 7S gave an average value of 358 ± 12 nm, which agrees well with earlier estimates of 355–358 nm for this length in human and mouse tumor type IV collagen molecules (19, 26). Of the 85 arms examined, 65 possessed a IA8 antibody molecule located at the same site 288 ± 11 nm from the overlap of the 7S domain. For the $(F1)_2F2$ fragment to be located farthest from the 7S domain, then the difference in length between the binding site of the antibody and the full length of the triple helix (i.e., 358 − 288 nm or 70 nm) should correspond closely to one half of the length of $(F1)_2F2$ (74.5 nm). This occurs and provides direct evidence for the location of $(F1)_2F2$ along the major triple helical domain.

ANTIBODY ID2: Fig. 4 shows the binding of antibody ID2 to the 7S domains of several type IV collagen molecules isolated from lathyritic chicken gizzard after a very limited pepsin digestion. In all cases, several ID2 molecules were observed binding to one 7S domain that has become obscured by the antibody. The *inset* of Fig. 4 shows a type IV collagen "spider" with four intact arms in which the binding of two or possibly three antibody molecules to the 7S domain can be recognized. No other specific binding of the antibody could be recognized along the collagen triple helix. Of 38 of the 7S

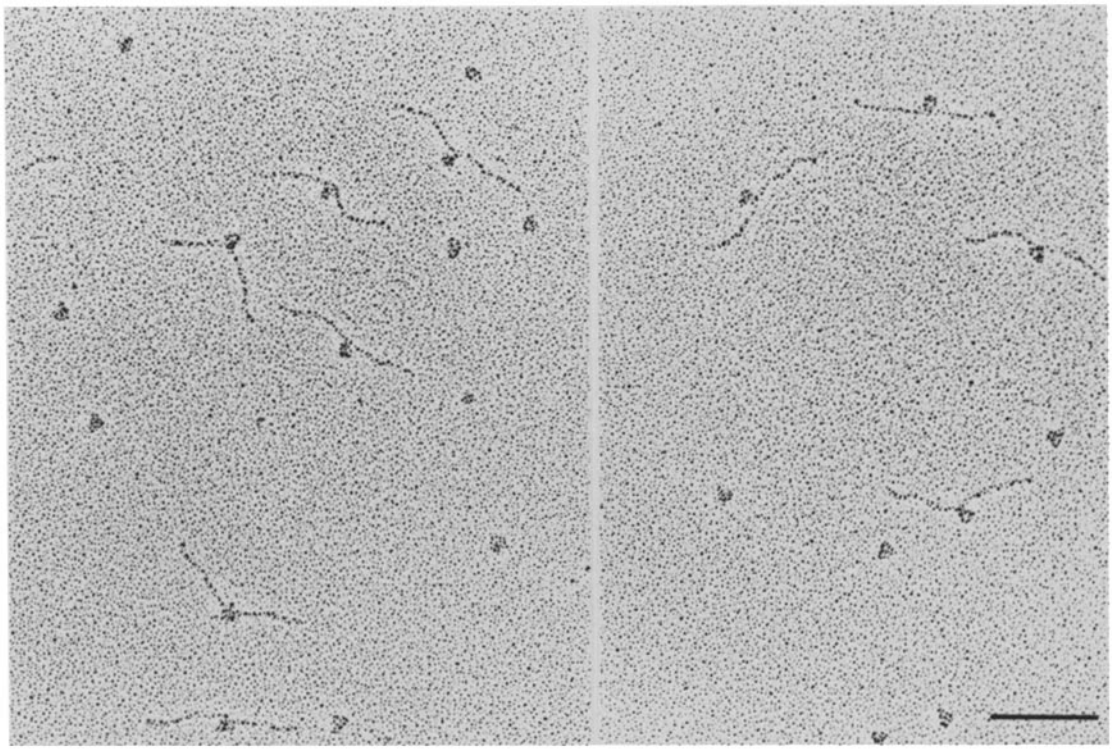

FIGURE 2 Electron micrograph after rotary shadowing of antibody IA8 interacting with the fragment of type IV collagen designated $(F1)_2F2$. The antibody (40 μg/ml) and the antigen (80 μg/ml) were mixed together and dialyzed overnight against 0.2 M ammonium bicarbonate before being sprayed onto freshly cleaved mica and shadowed with platinum. Bar, 100 nm. × 144,000.

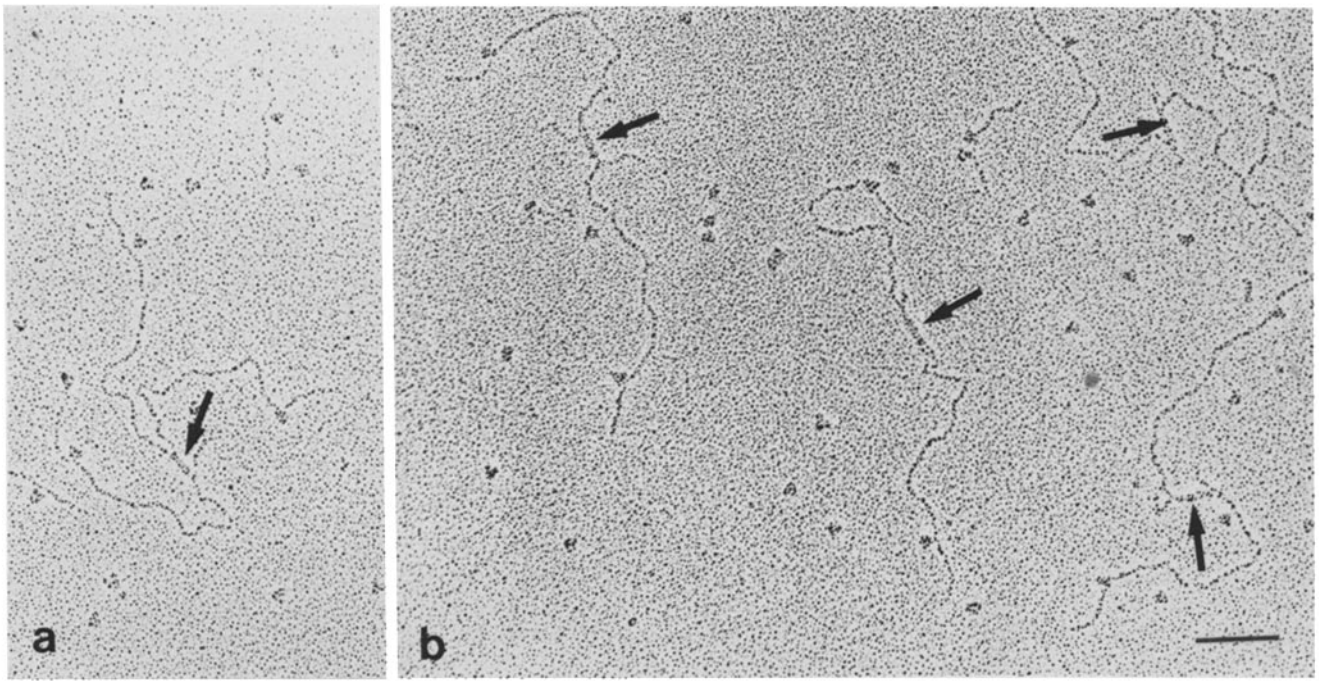

FIGURE 3 Electron micrograph after rotary shadowing of antibody IA8 interacting with a type IV collagen preparation isolated from lathyritic chicken gizzard by very limited pepsin digestion. The antibody and antigen were mixed together at concentrations of 40 μg/ml and 80 μg/ml, respectively. Note the presence of 7S domains (arrows) from which two or three full length collagenous arms extend and to which the binding of antibody IA8 can be observed at the expected location near the end. Also, note the occasional fragment of $(F1)_2F2$ with binding of IA8 at the center. Bar, 100 nm. × 116,000.

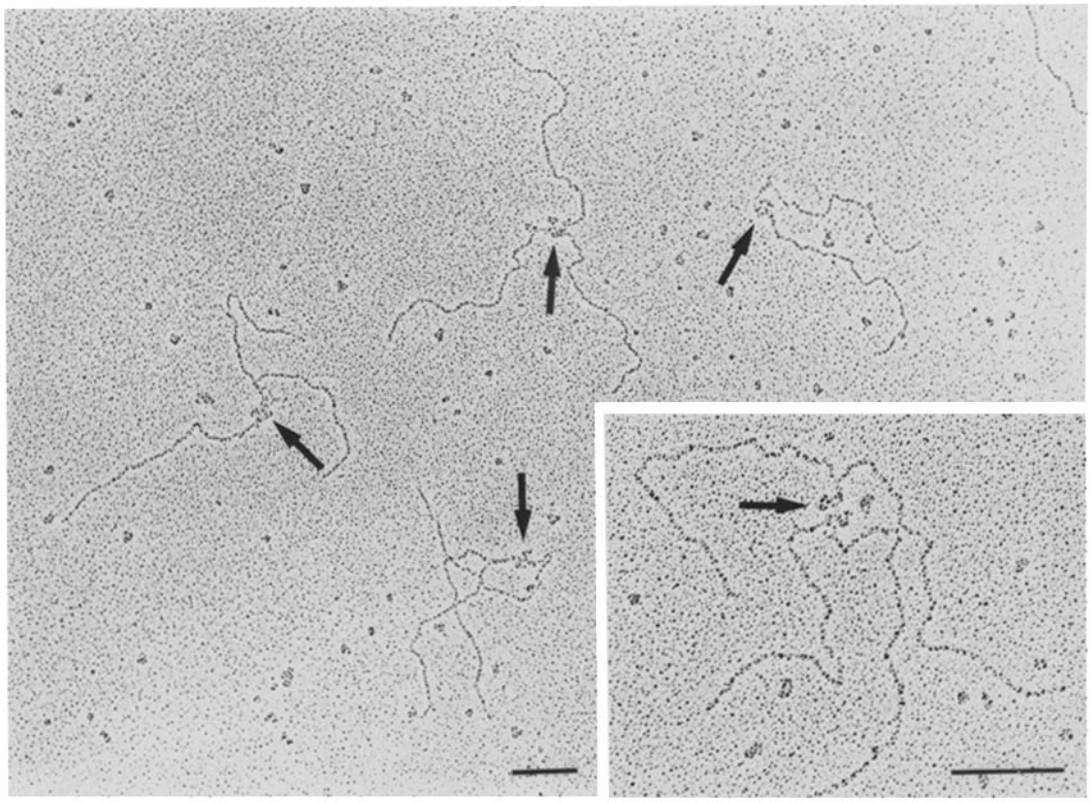

FIGURE 4 Electron micrograph after rotary shadowing of antibody ID2 interacting with a type IV collagen preparation isolated from lathyritic chicken gizzard by very limited pepsin digestion. The antibody and antigen concentrations were both 50 μg/ml. Note that several ID2 molecules are binding to each 7S domain. Bar, 100 nm, × 87,000. (*Inset*) A spider of four type IV collagen molecules in which binding of two or possibly three molecules of ID2 to the 7S domain can be clearly observed. Bar, 100 nm. × 150,000.

domains that were examined, 35 possessed at least one ID2 molecule binding to this site.

ANTIBODY IIB12: In Fig. 5*A* a single IIB12 antibody molecule is observed binding to one of the four arms of a type IV spider extending from 7S, whereas in Fig. 5*B* three antibodies are present, all apparently binding equidistant from the 7S domain. One of these latter antibodies may be cross-linking two of the arms extended from 7S. These results were obtained using a very low ratio of antibody to antigen (30 μg/ml antibody mixed with 50 μg/ml antigen), but with a higher ratio of antibody to antigen (50 μg/ml and 50 μg/ml) additional associations of antibody molecules to the type IV spiders were observed. Fig. 5*C* shows a histiogram of the distribution of antibody molecules along each spider. The results clearly show that specific binding and not random apparent associations occurred only in the region close to 7S, and it was estimated that the location of the epitope for IIB12 is 73 ± 8 nm (72 measurements) from the overlap of 7S. For this calculation only those antibodies that bound between 60 and 89 nm from the overlap of 7S (62% of all antibody binding) were included in the calculation.

These results on the immunolocalization of the three monoclonal antibodies to type IV collagen have made it possible to construct an "immunomap" of a spider as illustrated in Fig. 6. Four identical type IV molecules are considered to overlap in an antiparallel manner at the 7S domain, and as many as four epitopes will therefore be available for binding of ID2 at this site. Previous data suggested that the F3 domain is closest to 7S (11, 17), and binding of IIB12 occurs within the proposed location of this fragment. Finally, the rotary shadowing observations clearly show that $(F1)_2F2$ is located farthest from 7S. In the diagram the noncollagenous carboxyl termini (27), called NC-1 (26), have also been included, although in our preparations these are cleaved during pepsin digestion to prepare the spiders.

Localization of the Epitopes for Monoclonal Antibodies to Type V Collagen

ANTIBODY DH2: In Fig. 7 three molecules of type V collagen are shown with antibody DH2 bound to the same

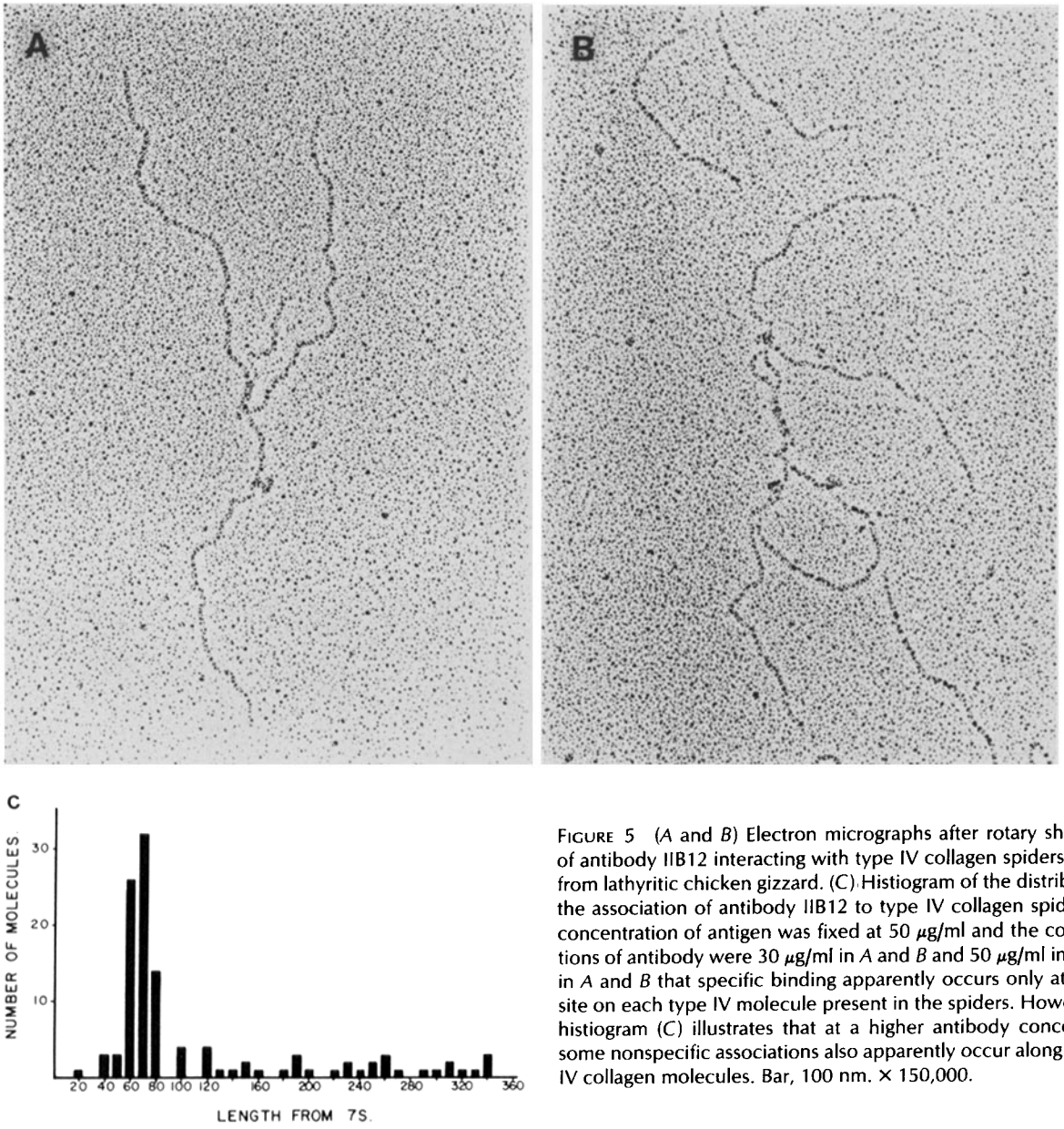

FIGURE 5 (*A* and *B*) Electron micrographs after rotary shadowing of antibody IIB12 interacting with type IV collagen spiders isolated from lathyritic chicken gizzard. (*C*) Histiogram of the distribution of the association of antibody IIB12 to type IV collagen spiders. The concentration of antigen was fixed at 50 μg/ml and the concentrations of antibody were 30 μg/ml in *A* and *B* and 50 μg/ml in *C*. Note in *A* and *B* that specific binding apparently occurs only at a single site on each type IV molecule present in the spiders. However, the histiogram (*C*) illustrates that at a higher antibody concentration some nonspecific associations also apparently occur along the type IV collagen molecules. Bar, 100 nm. × 150,000.

site located close to one end of each molecule. Of 91 molecules examined 56 (62%) were found to possess an antibody located at a distance of 45 ± 8 nm from one end of the molecule. No other specific binding site could be identified. We therefore propose that this site is the location of the epitope for DH2. The average length of the type V collagen molecules examined was 296 ± 8 nm (31 measurements), this comparing closely to the length of interstitial collagens.

ANTIBODY AB12: Fig. 8 shows that antibody AB12 appeared to bind to type V collagen at the same site as DH2, which was measured to be located 48 ± 5 nm from one end of the molecule. However, of 121 molecules examined only 54 (45%) of the molecules possessed an AB12 antibody binding at this site. The average length of the type V collagen molecules examined in this group was 292 ± 8 nm (42 molecules measured).

These results were surprising as in previous experiments ELISAs performed with a mixture of AB12 and DH2 gave partially additive results when both antibodies were present together as compared to each antibody being present separately (12). Experiments were therefore performed in which rotary shadowing was performed with a mixture of AB12 and DH2. Fig. 9 shows type V molecules with both antibodies

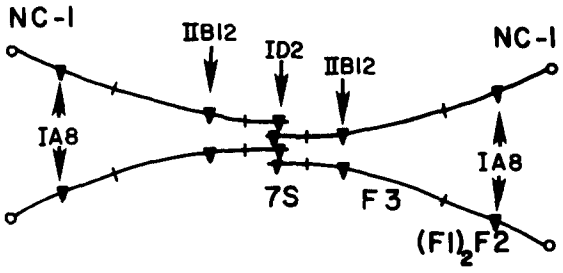

FIGURE 6 Immunomap of the location of the epitopes for monoclonal antibodies IIB12, ID2, and IA8 within a spider of type IV collagen. Note that four identical type IV molecules overlap at the 7S domain, and that the location of the epitopes for the three antibodies demonstrates that the three pepsin-resistant fragments are in the order proposed earlier (11, 17). NC-1 domains (26) have also been included in the model, although in the collagen preparations used in the present experiments these were cleaved away during pepsin digestion.

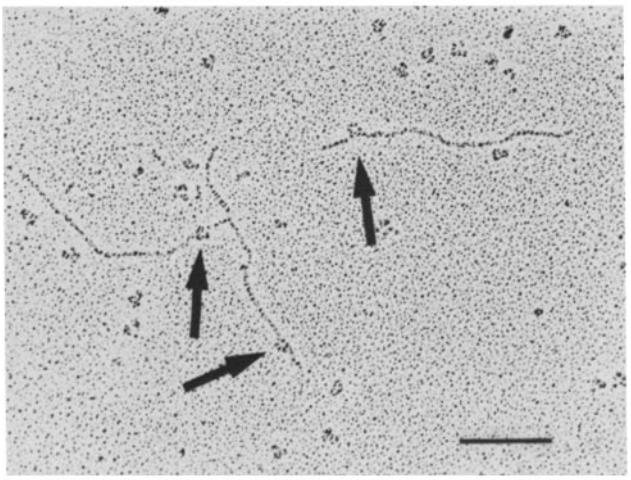

FIGURE 7 Electron micrograph after rotary shadowing of antibody DH2 interacting with type V collagen. Antibody and antigens were both at concentrations of 50 μg/ml. Note that the antibody is located at the same site for all three molecules. Bar, 100 nm. × 116,000.

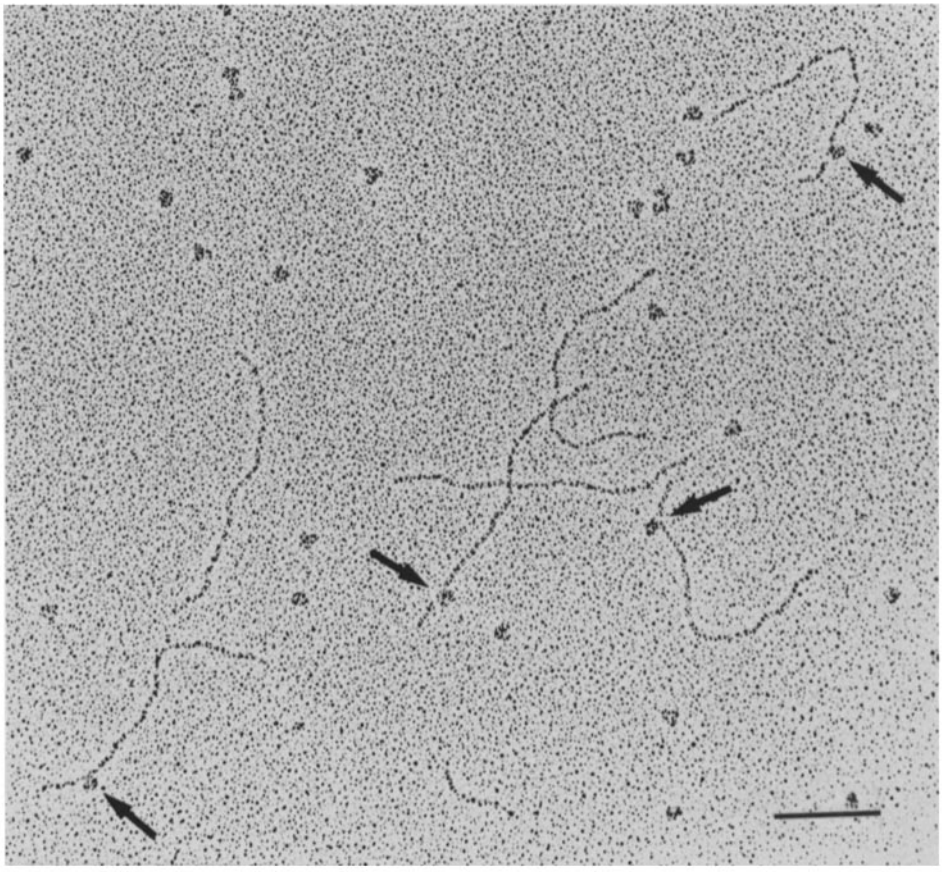

FIGURE 8 Electron micrograph after rotary shadowing of antibody AB12 interacting with type V collagen. Antibody and antigens were both at concentrations of 50 μg/ml. Arrows show the antibody binding to four molecules at the same site. Bar, 100 nm. × 145,000.

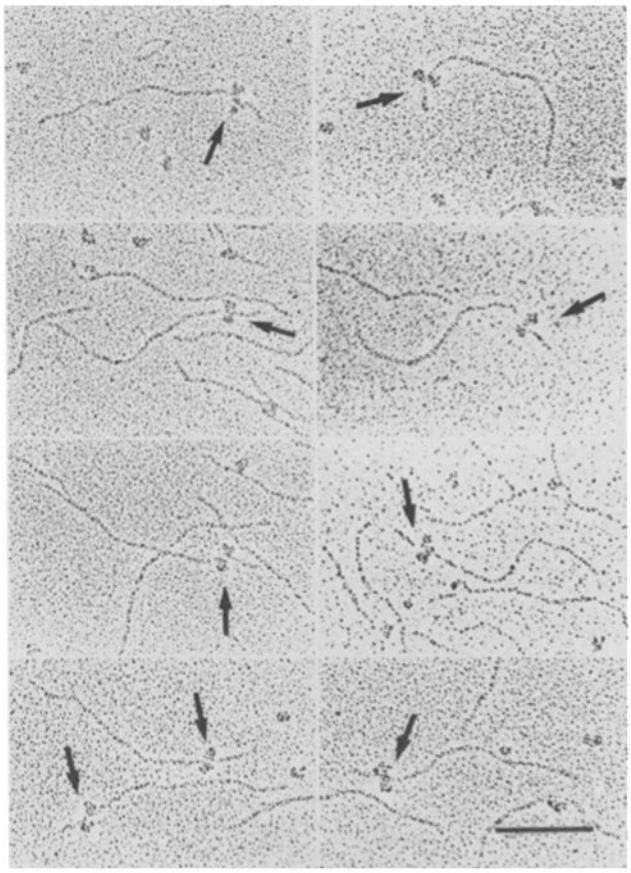

FIGURE 9 Composite electron micrographs of rotary shadowing of type V collagen in the presence of a mixture of antibody DH2 and AB12. The antibodies AB12 and DH2 were first mixed together in a 2:1 proportion, and then 50 μg/ml of this solution was mixed with 50 μg/ml of the antigen before rotary shadowing. Note the presence in each molecule (arrows) of two antibodies binding to the same site. Bar, 100 nm. × 130,000.

now located at the same site. Although the numbers of such molecules was small (30 of 629 molecules examined), such pairs of antibody molecules were never observed after rotary shadowing when either AB12 or DH2 were present separately. No binding of antibody 317 against human fibronectin could be observed at this site and, as a further control, rotary shadowing of type V molecules was also performed in the presence of antibody IIB12 (against the F3 fragment of type IV). No specific binding of this latter antibody could be observed, showing that there must be a unique interaction between both DH2 and AB12 at the same location along the type V molecule. We therefore conclude that the epitopes for DH2 and AB12 are very close to each other, and appear to map at the same site.

DISCUSSION

The present results show that it is possible to select monoclonal antibodies that recognize a single epitope in a collagen molecule with a sufficiently strong affinity that the interaction can be visualized in the electron microscope after rotary shadowing. Success with this approach has already been achieved with monoclonal antibodies to human type IV collagen (28, 29), and with a polyclonal antibody to bovine 7S (30). Rotary shadowing observations with one of the monoclonal antibodies to type IV collagen has shown that the epitope for this antibody is located 60 nm from the end of the overlap of 7S (28). However, human type IV collagen is not cleaved by pepsin into three native fragments in the same manner as the chicken, and so the type of immunomapping described in this paper has therefore not been possible.

The results in this paper now firmly establish that the order of the pepsin-resistant fragments of chicken type IV collagen is 7S-F3-$(F1)_2F2$, as suggested by earlier experiments (11, 17). In addition, the results provide further support to earlier proposals that there is a single type IV collagen molecule of chain organization $[\alpha1(IV)]_2\alpha2(IV)$ (16, 20). For chicken type IV collagen, this conclusion was previously based on extensive biochemical analysis of the fragment $(F1)_2F2$ for which after denaturation F1 and F2 were consistently isolated in a 2:1 proportion (16, 20). In this paper, we have observed that >90% of the $(F1)_2F2$ fragments are recognized by IA8, strongly suggesting that there is a homogeneous population of these fragments. In addition, the location of this fragment within the type IV collagen molecule is now clearly demonstrated.

Previous studies have established for mouse procollagen that the carboxyl termini of four type IV molecules that form the spider are located farthest from 7S (27) at a region called NC-1 (26). Four identical amino termini must therefore overlap to form the 7S domain. This conclusion is supported by the present observation of several ID2 molecules binding to a single 7S domain (Fig. 4). Previously, it has been shown that the additional cysteine residues of the $\alpha1(IV)$ chain which are not present in 7S are located towards the amino end of the major collagen triple helix (20, 31). The location of these residues presumably corresponds to the location of the interchain disulfide bridges present in F3, although at present there is no direct evidence to confirm this. A more direct demonstration of the location of the interchain disulfide bridges along the major triple helix of the type IV molecule has recently been achieved with a monoclonal antibody against native human type IV collagen (29). Rotary shadowing observations have shown that this antibody recognizes an epitope located 57 nm from the overlap region of 7S. The antibody also recognizes a small native fragment generated by CNBr cleavage of type IV collagen that contains $\alpha1(IV)$ CB15 and in which interchain disulfide bridges are present (32).

The present results now provide direct evidence that each monoclonal antibody against type IV collagen recognizes a single epitope present at different locations along the collagen molecule. It may be possible now to use these antibody molecules to perform electron microscopic immunolocalization of the different regions of the type IV molecule within basement membrane structure. Previous experiments with polyclonal antibodies against type IV collagen and electron microscopic immunolocalization have suggested that type IV collagen is present either in the lamina densa (33) or throughout the basement membrane of the glomerulus (34–36). It may now be possible to use our monoclonal antibodies for electron microscopic studies and show the location of the 7S, F3, and $(F1)_2F2$ domains in the glomerular basement membrane. With the two monoclonal antibodies to type V collagen, electron microscopic immunolocalization may also provide further information with regard to the function of this collagen. The present information from our light microscopic studies (12) and from electron microscopic immunolocalization with a polyclonal antibody to type V collagen (37) suggest that this collagen is present in interstitial connective tissue

(12). In the cornea it appears to be closely associated with the larger fibers of type I collagen (Fitch, J. M., J. Gross, R. Mayne, B. Johnson-Wint, and T. F. Linsenmayer, 1984, *Proc. Nat'l Acad. Sci. USA*, in press).

The significance of the localization of the epitopes for the two monoclonal antibodies to type V collagen to the same site along the type V molecule is at present unclear. It may be that there is a discrete region along the type V molecule that is highly immunogenic and gives rise to antibodies of sufficient affinity to survive our screening procedures. The site is located 45–48 nm from one end of the molecule or an approximated distance of 15% of the total length of the collagen molecule. This location appears to correspond closely in distance to the site of cleavage of type V collagen either with metalloproteinases that degrade native type V collagen (38–40) or with α-thrombin (41). However, we do not at present know if the epitopes and the cleavage site are both located at the same end of the molecule, and are either at, or very close to, the same site. To demonstrate this directly, it may be possible to inhibit protease cleavage of type V collagen with one or both of our antibodies. Similar experiments have recently been successfully performed with a monoclonal antibody against the amino terminal cleavage site of type I procollagen in which it was shown that the antibody would inhibit the activity of procollagen N-proteinase (42).

Our present method of immunomapping with monoclonal antibodies may also provide important structural information for other poorly characterized collagen molecules such as the minor cartilage collagens of high and low molecular weight which so far have only been isolated as pepsin-resistant fragments (21). It may also be possible to use monoclonal antibodies combined with rotary shadowing to investigate the macromolecular organization of collagen molecules that form complex structures too large to be analyzed directly by biochemical methods.

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